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Characterization of tributyrin hydrolysis by immobilized lipase on woolen cloth using conventional batch and novel spinning cloth disc reactors

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20 ## Abstract

21 Optimal loading and operating conditions for a new, superior immobilization of amano lipase
22 from *P. fluorescens* on woolen cloth were determined. The optimal enzyme loading was 46.8
23 mg g dry cloth^{-1} with activity of 200 U. A batch reactor was used to characterize process
24 conditions important to industrial application of the wool immobilized lipase. The optimal pH
25 for immobilized lipase in tributyrin hydrolysis was 7, slightly lower than that of free lipase
26 (pH 8). The optimal temperature for both free and immobilized lipase was 45 °C. The
27 immobilized lipase was more stable to reuse than some other lipase immobilizations,
28 maintaining 85% of its activity after 6 long term runs and 75.8% of the original activity after
29 storage of 40 weeks at 4 °C. The thermal stability of lipase was improved by 2.4 times after
30 immobilization. The thermal deactivation rate of immobilized lipase followed the Arrhenius
31 law with E_d=199 kJ mol^{-1}. The Michaelis-Menten constant (K_m) of the lipase increased from
32 1.63 mM to 4.48 mM after immobilization. The immobilized lipase was also successfully
33 applied for tributyrin hydrolysis in a novel enzyme process intensification technology – the
34 spinning cloth disc reactor (SCDR): conversion increased by around 13% under similar
35 conditions compared to a conventional batch stirred tank reactor. The SCDR is therefore key
36 to exploiting the advantages of the wool immobilized lipase developed in this work.

37

38

39 *Keywords:* enzyme immobilization; lipase; woolen cloth support; thermal deactivation;
40 tributyrin hydrolysis; spinning cloth disc reactor.

41

42 **1. Introduction**

43 Enzymes have advantages over most non-biological catalysts such as: high efficiency,
44 specificity and selectivity, and ability to function under mild conditions (Klibanov, 1983).
45 However, the main disadvantages of free enzymes are insufficient stability and difficulty of
46 recycling, restricting their wider industrial application compared to chemical catalysts
47 (Klibanov, 1983; Sheldon, 2007). Immobilization of enzymes has been shown to help
48 overcome these disadvantages; however the properties of the support and immobilization
49 methods have a significant effect on the activity of immobilized enzymes (Yemul and Imae,
50 2005). The properties of the support materials can affect the adsorption, conformation and
51 expressed activity of immobilized enzymes. In addition, the surface chemistry and size can
52 influence the behavior of enzymes and substrate at the support interfaces (Talbert and
53 Goddard, 2012). Although various immobilization protocols have been reported in the
54 literature, many of them include complex immobilization procedures as well as expensive
55 materials. Simple, effective and non-expensive immobilization methods are still very
56 desirable from both an academic and industrial/application point-of-view.

57 Lipases, known as triacylglycerol acylhydrolases, catalyze the hydrolysis of triglycerides to
58 fatty acids and glycerol over an oil-water interface (Treichel et al., 2010). In addition, in the
59 presence of lipases, the reverse esterification and transesterification reactions can occur in
60 water restricted environments (Al-Zuhair, 2005). Due to their ability to catalyze reactions
61 with high specificity and selectivity, lipases have been widely used in food (Othman et al.,
62 2008), organic synthesis (Gomes et al., 2004) and dairy industry (Ren et al., 2008). Several
63 methods have been reported for the immobilization of lipases on different supports, including
64 covalent binding (Ye et al., 2005), encapsulation (Yang et al., 2009) and adsorption (Mateo et
65 al., 2000). However, it is still a challenge to obtain high protein loading and enzyme activity
66 for practical applications. A variety of materials have been used as the support for enzyme

67 immobilization. Among them, fibers have been increasingly investigated due to their low
68 price, large specific surface and excellent mechanical properties. For example, cotton, silk
69 and nylon have been reported to be successfully employed for enzyme immobilization
70 (Albayrak and Yang, 2002; Chatterjee et al., 2009; Isgrove et al., 2001). Wool, as a complex
71 and highly cross-linked protein fiber, consists of keratin type proteins that have rich reactive
72 residues thus giving it great potential to be used as immobilization support. Although wool is
73 a very important biomaterial, it has not been well studied like other fabrics in enzyme
74 immobilization. To date, lipase has been reported to be immobilized on to wool in a small
75 number of studies (An et al., 2008a; An et al., 2008b; Monier et al., 2010). However, several
76 drawbacks to these techniques limited its practical application, including low stability and the
77 necessary use of complex immobilization procedures. Recently, a simple and effective
78 protocol has been reported by the authors to immobilize lipase onto woolen cloth (Feng et al.,
79 2013a). Lipase was immobilized on polyethyleneimine (PEI) modified woolen cloth with
80 glutaraldehyde (GA) as a cross linker. The success of the immobilization was verified by
81 means of zeta potential, FTIR and confocal laser scanning microscope. A chemical analysis
82 of immobilized lipase was thoroughly performed, showing that the enzyme loading is mainly
83 determined by the electrostatic interaction between lipase and woolen cloth, and the optimal
84 pH for immobilization is around 6. The current paper will focus on investigating the
85 performance of immobilized lipase in reactions to determine its suitability for industrial
86 application.

87 For immobilized enzymes to be adopted by industry, they must also be applied in a reactor,
88 which can produce a stable, high yield and fast reaction. There are many reactors available,
89 but this paper will look at applying the immobilized lipase on wool to two types only: (1) a
90 conventional batch stirred tank reactor (BSTR) to determine the conventional performance of
91 the immobilized lipase and, (2) a new innovation in enzyme reactor technology – the

92 spinning cloth disc reactor (SCDR) - which is a variant on the more common spinning disc
93 reactor (SDR) used for process intensification. In the SCDR (Fig. 1), a liquid stream is fed
94 onto the top of a spinning disc which holds the lipase immobilized onto wool on top. The
95 centrifugal force of the spinning disc forces this liquid onto and into the wool, forming a
96 highly sheared thin film on top of and within the rotating cloth. Research has shown for
97 conventional SDRs that the heat and mass transfer can be significantly enhanced by the fluid
98 dynamics within these films (Jachuck and Ramshaw, 1994; Meeuwse et al., 2012; Visscher et
99 al., 2012) resulting in process intensification, where the reaction rates are much higher than
100 conventional reactors operated under comparable conditions (Boodhoo and Jachuck, 2000).
101 Therefore it would be interesting to investigate if such advantages can be extended into a
102 cloth immobilized enzyme system.

103 Consequently, the aims of this study are:

104 1. To determine the optimal enzyme loading (in terms of activity) for the wool
105 immobilized lipase.
106 2. To characterize and compare key performance characteristics of the wool
107 immobilized lipase and free lipase: thermal stability, pH stability, and reaction
108 kinetics.
109 3. To determine the operational stability of the wool immobilized lipase by evaluating
110 the impact of reuse on activity.
111 4. To compare the performance of this immobilized lipase in two types of enzyme
112 reactors: a conventional BSTR and a novel SCDR.

113

114 **2. Materials and Methods**

115 **2.1. Materials**

116 Unbleached organic woolen cloth was bought from Treliske (Otago, New Zealand). Amano
117 lipase from *P. fluorescens*, polyethyleneimine (PEI), tributyrin (98%), triton X-100,
118 Coomassie brilliant blue G 250, sodium bicarbonate and sodium carbonate were obtained
119 from Sigma-Aldrich (New Zealand). Glutaraldehyde (GA) 25% (w/v), sodium dihydrogen
120 phosphate and disodium hydrogen phosphate were purchased from Unilab (ECP, New
121 Zealand). Hydrogen peroxide 30% (v/v) was obtained from Scharlau (Thermofisher, New
122 Zealand). Bovine serum albumin (BSA) was obtained from Gibcobrl (Life Technologies,
123 New Zealand). All chemicals were used as received. All solutions were prepared using
124 deionized water (produced from a Milli-Q Gradient A10, Millipore).

125 **2.2. Pretreatment of the woolen cloth**

126 The woolen cloths were cut into 4×4 cm squares and treated with a solution containing 30
127 mL L^{-1} hydrogen peroxide (30%) and 2 g L^{-1} sodium silicate at pH 9 (0.1 M Na_2CO_3,
128 $NaHCO_3$ buffer) at 55 °C for 70 min. The treated cloths were thereafter thoroughly rinsed
129 with deionized water three times and air dried.

130 **2.3. Enzyme immobilization**

131 The detailed procedure of enzyme immobilization has been described in the authors' recent
132 publication (Feng et al., 2013a). The main process was as follows: the bleached woolen cloth
133 was firstly immersed in 2% PEI solution at pH 8 for 2 h at room temperature and then rinsed
134 with deionized water. The resulting cloth was soaked in lipase solution (0.1 M phosphate
135 buffer, pH 6) with different concentrations for 24 h, followed by immersion in 0.5% (w/v)
136 GA solution (0.1 M phosphate buffer, pH 6) for 10 min for cross-linking, unless stated

137 otherwise. The cloth was then washed with deionized water until no free enzyme was
138 detected in the washed solution. The immobilized lipase was stored in a pH 7, 0.1 M
139 phosphate buffer solution at 4 °C until further use.

140 **2.4. Enzyme loading determination**

141 The enzyme loading on woolen cloth was determined by measuring the protein content of the
142 enzyme solution after immobilization and in the washed solution using the Bradford method
143 at 595 nm using bovine serum albumin (BSA) as the standard (Bradford, 1976).

144 **2.5. Application of wool immobilized lipase in the BSTR and lipase activity assay**

145 Tributyrin hydrolysis in a conventional BSTR with a pH stat (807 Dosing unit, Metrohm,
146 Switzerland) was used as the benchmark reactor for this work. All lipase activity
147 measurements were performed in this system except for the SCDR experiments. The activity
148 of free and immobilized lipase were both determined. This system is the same as that in Fig.
149 1, except there was no spinning cloth disc reactor and the pump inlet and outlet, and cloth
150 was placed directly in the reactant vessel. In a typical experiment, 0.33 g tributyrin and 0.15 g
151 triton X-100 were added to phosphate buffer solution (pH 7) to make a final volume of 85
152 mL. This substrate solution was agitated with a magnetic stirrer at 25 °C at 600 rpm for 30
153 min to form a homogenous emulsion. The reaction was then started by adding either free or
154 immobilized lipase and monitored for 10 min in a water bath at 45 °C. During the hydrolysis,
155 sodium hydroxide was added into the reactor by the pH stat to neutralize the produced fatty
156 acid. Each reaction was repeated at least 3 times and error bars were calculated as ± one
157 standard deviation. One enzyme unit (U) was defined as the amount of lipase which catalyzes
158 the release of 1 μmol butyric acid per minute under the specified conditions. Reaction
159 conversion was correlated to moles of sodium hydroxide consumed by the reaction according
160 to Eq. 1:

$$Conversion(\%) = \frac{moles\ of\ free\ butyric\ acids}{moles\ of\ original\ esters\ in\ tributyrin} \times 100 \quad (1)$$

2.6. Thermal stability of free and immobilized lipase

Free and immobilized lipase preparations were incubated in pH 7 phosphate buffer solution at 60˚C and sampled periodically. The residual activities were evaluated by the tributyrin emulsion method as described in Section 2.5. The hydrolytic activity of the non-incubated enzyme was taken as 100%.

2.7. Thermal deactivation of immobilized lipase

Heat is one of the most important causes of enzyme deactivation in industrial reactors (Klibanov, 1983; Luo and Zhang, 2010). Therefore, it is of vital importance to investigate the deactivation kinetics of the immobilized lipase to provide valuable information for further industrial application. Many theoretical mechanisms and mathematical models have been proposed to describe the thermal deactivation of enzymes. Among them, a general scheme applicable to the deactivation of most enzymes was developed by Henley and Sadana (1986). A first order process has been most widely used in enzyme thermal deactivation due to its simplicity (Peterson et al., 1989):

$$E \xrightarrow{k_d} E_d \quad (2)$$

Where E is the active enzyme; E_d is the deactivated enzyme; k_d is the deactivation rate constant.

Following this process, the thermal deactivation rate equation can be obtained:

$$\frac{da}{dt} = -k_d a \quad (3)$$

Where a is the enzyme activity at time t during the thermal deactivation process.

Eq. 3 can be integrated to Eq. 4:

183 $$\ln\left(a / a_0\right) = -k_d t \tag{4}$$
184
185 Where a_0 is the initial activity.
186
187 Therefore, at a given temperature, a semi natural logarithm plot of residual activity versus
188 time should give a straight line where the negative slope is the deactivation rate constant k_d.

189 The Arrhenius equation has been widely used to describe the relationship between the
190 reaction rate constant and temperature. Therefore, k_d can then be expressed as follows:

191 $$k_d = A_d e^{-E_d / RT} \tag{5}$$

192 Where E_d is the thermal deactivation energy; A_d is the pre-exponential factor.

193 The natural logarithm of k_d versus reciprocal absolute temperature should give a straight line,
194 where E_d and A_d can be determined from the slope and intercept.

195 To characterize the thermal deactivation of the wool immobilized lipase, the immobilized
196 lipase was incubated in a water bath at 60, 65 and 70 ˚C respectively. Samples were taken
197 periodically and the activity was tested as described in Section 2.5.

198 **2.8. Application of wool immobilized lipase in the SCDR**

199 A schematic diagram of the batch SCDR process used in this study is shown in Fig. 1a. The
200 SCDR consisted of the pH stat, a liquid feeding system, an overhead stirrer connected to a
201 disc, a liquid funneling vessel around the disc, and a reactant solution storage vessel. The
202 critical spinning surface in this SCDR was a Perspex disc 250 mm in diameter, driven by a
203 variable speed motor (Glas-Gol, US). This spinning disc was enclosed in a steel funnel-
204 shaped chamber 300 mm in diameter and 210 mm deep. Woolen cloth was cut into circular
205 pieces 250 mm in diameter and used as support for lipase immobilization according to the
206 procedure described in Section 2.3. Then this woolen cloth with immobilized lipase was fixed
207 on the disc as shown in Fig. 1b. Further details can be obtained from Feng et al., (2013b). In a

208 typical experiment, firstly, the disc with immobilized lipase woolen cloth was connected to
209 the driving motor and spun to the desired rotational speed. Then, the reaction was launched
210 by pumping the tributyrin emulsion to the center of the spinning disc. As a result, the solution
211 was spread over the spinning cloth surface (and within the volume of the cloth due to
212 wetting) by the centrifugal force. The tributyrin was hydrolyzed by the immobilized lipase on
213 the cloth, and thereafter returned to the feed vessel. During the hydrolysis, the volume of
214 sodium hydroxide added into the feed vessel by the pH stat to keep a constant pH was
215 measured. All results were repeated three times and error bars were calculated as ± one
216 standard deviation.

217 **3. Results and Discussion**

218 **3.1. Optimizing immobilized enzyme loading and activity: the effect of lipase**
219 **concentration during immobilization**

220 For a successful enzyme process at industrial scale, obtaining the highest reaction rate per
221 area of support used in a reactor is key to obtaining an economically feasible reaction.
222 Consequently, the enzyme loading and associated activity on the wool support needs to be
223 maximized. Fig. 2 shows the enzyme loading and activity as a function of the amount of
224 lipase in the immobilization solution ranging from 0.5 to 5 g L^{-1}, with lipase activity
225 measured in the BSTR system as described in Section 2.5. It can be seen that both the enzyme
226 loading and activity increased with the increase of lipase provided. The maximum activity
227 (215.6 U g $cloth^{-1}$) and enzyme loading (61.98 mg g $cloth^{-1}$) were obtained with a lipase
228 concentration of 5 g L^{-1}. When the lipase concentration was increased from 0.5 to 2 g L^{-1}, the
229 activity and enzyme loading showed a dramatic increase of 90.6% and 214.4% respectively.
230 Thereafter (between 2 and 5 g L^{-1},) the increase of activity was less significant (increased by
231 7.8%). However, the enzyme loading kept increasing until the lipase concentration reached 3

232 g L^{-1} (increased by 25.5% from 2 to 3 g L^{-1}), indicating that the activity did not correspond
233 strictly to enzyme loading on wool and instead it may also be dependent on steric effects. In
234 view of these results, 2 g L^{-1} of lipase (producing an enzyme loading of 46.8 mg g $cloth^{-1}$)
235 was used in the immobilization protocol for all the following experiments due to this being
236 around the optimum, giving high activity and immobilization efficiency.

237 **3.2. Free vs. wool immobilized lipase 1: Effect of pH and temperature on activity**

238 The operating envelope of the immobilized enzyme needs to be well defined so that a well-
239 controlled and robust reaction process can be designed and used in practice. Two key
240 variables during the operation of any enzyme reaction and reactor are pH and temperature, as
241 adverse values of these will deactivate the enzymes. Consequently, the pH and temperature
242 profiles of activities of both free and immobilized lipase were quantified.

243 Fig. 3 depicts the pH profile of the activity of both the free and immobilized lipase. The free
244 lipase displayed a maximum activity at around pH 8, which is consistent with data from the
245 product supplier (Sigma-Aldrich). The optimum pH for immobilized lipase was reduced
246 approximately 1 pH unit from 8 to 7 compared to the free lipase. This change in optimum pH
247 might be due to a change in the charge distribution of the functional amino acids of the lipase
248 after immobilization. The modification with PEI introduces more alkaline groups to the lipase
249 surface during immobilization and provides an alkaline environment which is more favorable
250 for tributyrin hydrolysis, shifting the optimum pH to a more acidic value. In earlier studies
251 where PEI was also used for enzyme immobilization, similar changes in optimum pH were
252 observed, validating the results in this study (Gao et al., 2006; Kamath et al., 1988; Yun et al.,
253 2000).

254 The effect of temperature on the activity of both free and immobilized lipase is given in Fig.
255 4. The results demonstrated that the wool immobilization of lipase did not change its activity

256 vs temperature profile. The activity of both lipase forms increased dramatically as the
257 temperature increased from 25 °C to 45 °C, because the higher temperature was able to not
258 only accelerate the diffusion of lipase and substrate (thus leading to a shortening of the
259 contact time required for reaction to take place), but also overcome the activation energy
260 barriers allowing a higher enzyme activity. After the temperature reached 45-55 °C, the
261 activity decreased with a further increase in temperature due to the thermal deactivation of
262 the lipase. Therefore the immobilized enzymes should be used at 55 °C and below to avoid
263 decreased activity, defining the top end of the thermal operating envelope in the BSTR and
264 SCDR.

265 **3.3. Free vs. wool immobilized lipase 2: thermal stability over time**

266 The thermal stability with time of both free and immobilized lipase were investigated at
267 60°C. The free lipase lost most of its activity more rapidly than the immobilized lipase: a
268 relative activity of 29% remained for free lipase after 300 min but 70% for immobilized
269 lipase after 390 min (Fig. 5). It has been reported that the thermal stability could be largely
270 improved by immobilization due to the enhanced tertiary structure stability upon the covalent
271 binding of enzymes to the support (Bai et al., 2006; Bayramoglu et al., 2005; Ghamgui et al.,
272 2007; Yemul and Imae, 2005).

273 **3.4. Thermal deactivation kinetics of the wool immobilized lipase**

274 Thermal deactivation rates at 60, 65 and 70 °C for the wool immobilized lipase were
275 quantified to better understand the stability phenomena presented in Fig. 4 and Fig. 5, and to
276 estimate deactivation kinetics. As shown in Fig. 6, the residual activity of the immobilized
277 lipase decreased with incubation time at all three temperatures, further confirming that
278 thermal deactivation occurred. The semi-log plot of residual activity (Fig. 6) showed a
279 reasonably good linear relation with incubation time at various temperatures, indicating that
280 the deactivation of this immobilized lipase on woolen cloth can be described by first order

281 kinetics. This means that from Eq. 4, the half-life $t_{1/2}$ for the immobilized lipase can also be

282 calculated (using $\ln 2/k_d$). The deactivation rate constants at various temperatures and their

283 corresponding half-life are given in Table 1. The deactivation rate was faster at a higher

284 temperature. This is also clear from the data in Fig. 6: at 60 °C, only 30.2% of the original

285 activity was lost after 390 min, however at 70 °C, 79.7% of the initial activity was lost after

286 200 min. To determine the relationship between temperature and rate, the data was plotted to

287 determine a fit to Eq. 5, as shown in Fig. 7. This indicates that the deactivation kinetics for

288 the wool immobilized lipase follows an Arrhenius type relationship with temperature, and the

289 deactivation energy is approximately 199 kJ mol^{-1}. There are no comparable values for the

290 amano lipase used in this study, however the deactivation energy was 114.3 to 143.6 kJ mol^{-1}

291 for the lipase from *C. rugosa* immobilized on six different supports (Shaw et al., 1990) –

292 lower than in this study. The difference may just be due to the fact that the lipases are from

293 different microbial sources (so naturally have different deactivation energies). Indeed, the

294 deactivation energy value varies widely between different lipases: for example, 93.8 kJ mol^{-1}

295 for lipase from *P. citrinum* (Pimentel et al., 1997), 228.8 kJ mol^{-1} for lipase from *M.*

296 *javanicus* (Balcao et al., 1998), and 304 kJ mol^{-1} for lipase from *R. miehei* (Noel and

297 Combes, 2003). Our value is within the overall range reported.

298 **3.5. Free vs. wool immobilized lipase 3: initial rate kinetics**

299 Quantifying reaction kinetics is a key step in understanding how the reaction mechanism may

300 have been affected by the immobilization process, and reaction kinetics can also provide a

301 means and basis from which reactor sizing and design can be calculated. The Michaelis-

302 Menten initial rate kinetics of both free and immobilized lipase for tributyrin emulsion

303 hydrolysis were compared in the BSTR at different concentrations, ranging from 5 to 40 mM.

304 The initial rate was estimated by means of the slope of the hydrolysis curve (produced by the

305 pH stat) at the beginning of the reaction: this method has been shown to accurately quantify

306 the initial rate (Jurado et al., 2006). Since the pH stat provides a large continuous data set
307 (logging pH and base addition every two seconds), a large number of experimental points
308 were incorporated into a least squares fitting method for a straight line, to provide an accurate
309 estimate of the initial rate. All data points from the pH stat were included from the start of the
310 reaction until the point at which the slope of the fitted straight line began to decrease, which
311 has previously been shown to be where the initial rate period ends (Haas et al., 1995).

312 The kinetic constants were evaluated using a Lineweaver-Burk plot, as shown in Fig. 8. The
313 Michaelis–Menten constant K_m of immobilized lipase was estimated to be 4.48 mM, which
314 was nearly threefold higher than that of the free lipase, which was 1.63 mM. An increase in
315 K_m after immobilization has been seen in other research: for example, Ye et al. (2005) found
316 that K_m of lipase increased from 0.45 to 1.36-1.43 mM after immobilization on a membrane
317 (Ye et al., 2005). Yiğitoğlu et al. (2010) immobilized lipase on polyester fibers and found that
318 the K_m increased from 47.2 to 151.6 mg mL^{-1} after immobilization (Yigitoglu and Temoçin,
319 2010). K_m is described as an inverse binding constant: an increase in the K_m value indicates
320 that immobilized lipase has a lower affinity to the substrate. This is likely due to either a
321 distortion of tertiary structure caused by immobilization and/or the expected mass transfer
322 resistances caused mainly by the larger stagnant film around the larger woolen cloth surface
323 compared to the free lipase.

324 **3.6. Storage stability of immobilized lipase**

325 Storage stability is another critical factor to be considered for the industrial application of
326 these wool immobilized enzymes. Enzymes must be able to retain their activity after
327 transportation and storage so that they can be used at near maximum (fresh) activity in the
328 industrial process. In this study, storage stability was investigated by storing the immobilized
329 lipase on woolen cloth in phosphate buffer (0.1 M, pH 7) for an extended period at two
330 different temperatures: 4°C and 25°C. As shown in Fig. 9, the immobilized lipase maintained

331 75.8% and 55.3% of its original activity after storage of 40 weeks at 4°C and 25 °C,
332 respectively. It is well accepted that a lower temperature is more favorable for enzymes to
333 maintain the tertiary structure, thus keeping a high activity. This result indicates that it is
334 possible to store the immobilized lipase for an acceptable period for industrial application
335 (i.e. for transportation and storage before application) with high residual activity.

336 **3.7. Operational stability of the wool immobilized lipase**

337 One advantage of immobilized enzyme over its free form is the reusability, so a successful
338 immobilized enzyme system should be reusable with both good stability and high activity.
339 Therefore, the loss of enzymatic activity during repeated use was investigated over 6 batches
340 of tributyrin hydrolyses by reusing the same cloth. After each consecutive 4 h run, the woolen
341 cloth with immobilized lipase was washed with phosphate buffer (pH 7, 0.1 M) and
342 reintroduced to the fresh tributyrin emulsion (13 mM) at 45 °C. Fig. 10 presents the
343 operational stability of the immobilized lipase over the 6 runs.

344 It can be seen that the tributyrin conversion was reduced 3% between the first and second run
345 which accounted for 32% of the total activity loss, and this can most likely be attributed to
346 the release of free lipase, which was most likely adsorbed onto the wool surface rather than
347 more strongly bound via the intended covalent binding (Feng et al., 2013a). The tributyrin
348 conversion decreased from 62.3% to 52.7% after 6 runs, which means that the immobilized
349 lipase maintained 85% of its original activity. This reusability is superior to previous studies
350 of wool in lipase immobilization, where, for example, there was less than 70% retained
351 activity after 6 cycles of 5 min each (Monier et al., 2010). This result is also better than that
352 seen with some other lipase immobilization methods. For example, it was reported that lipase
353 immobilized on insoluble yeast β-glucan maintained around 50% of its original activity after
354 6 reuses (Vaidya and Singhal, 2008). Özmen and Yılmaz (2009) immobilized lipase on β-
355 cyclodextrin-based polymer and retained 80% activity after 6 runs (Ozmen and Yilmaz,

356 2009). This result demonstrates that the protocol developed for wool in this study can achieve
357 a superior immobilization, and has good potential for application in a continuous lipase
358 reactor, such as for the continuous hydrolysis of tributyrin.

359 **3.8. Comparison of BSTR and SCDR performance**

360 All of the results above show that the wool immobilized lipase provides a highly active and
361 stable performance in a conventional BSTR. However, for wool to be considered a versatile
362 support material for lipase immobilizations, it should be able to function well in a range of
363 different reactors. Therefore the performance of the wool immobilized lipase was quantified
364 in a new type of enzyme process intensification reactor – the SCDR. Operation in the SCDR
365 is expected to be more testing than in a BSTR: the high shear forces produced by the
366 centrifugal movement of the reactant/product solution on the spinning disc are a more
367 adverse environment for immobilized lipase than a BSTR as they can potentially deactivate
368 under high hydraulic shear.

369 Fig. 11 shows the conversion of tributyrin with time in both the SCDR and BSTR at two
370 initial tributyrin concentrations (20 and 40 g L^{-1}). Under comparable reaction conditions,
371 tributyrin hydrolysis in the SCDR proceeded at a higher rate than in the BSTR, giving a
372 higher conversion over the entire hydrolysis process at all the investigated concentrations: for
373 example, for 20 g L^{-1} tributyrin, the final conversion was 52.2% in the BSTR and 65.4% in
374 the SCDR after 240 min. This result indicates that both the reaction rate and yield are
375 improved in the SCDR, which is most likely attributed to the more rapid mixing between
376 substrate and immobilized lipase, and enhanced mass transfer in the thin film on top of and
377 within the spinning cloth. This confirms that the wool immobilized lipase is a robust
378 immobilization system for this type of lipase and that the SCDR can intensify enzyme
379 reactions. This combined system is therefore worthy of a more detailed study and so a full
380 characterization of the SCDR will be performed.

381 **4. Conclusions**

382 Amano lipase from *P. fluorescens* has been successfully immobilized on woolen cloth using
383 polyethyleneimine (PEI) with glutaraldehyde (GA) cross-linking. The enzyme immobilized
384 on one gram cloth was 46.8 mg with activity of 200 U. The protocol developed for wool in
385 this study can achieve a superior immobilization, where the wool immobilized lipase
386 potentially has sufficient activity and stability to be an effective enzyme system for industrial
387 enzyme processes. A number of different parameters were optimized and quantified,
388 primarily in a conventional BSTR system:

389 - The optimal pH for immobilized lipase in tributyrin hydrolysis was 7 which was
390 slightly lower than that of the free lipase (pH 8), implying the introduction of PEI
391 provides an alkaline environment which is more favorable for tributyrin hydrolysis in
392 a more acidic value.
393 - The optimal temperature for both free and immobilized lipase was 45 °C.
394 - The thermal stability of lipase was significantly improved after immobilization. The
395 thermal deactivation rate of immobilized lipase was found to follow the Arrhenius law
396 with the thermal deactivation energy of 199 kJ mol^{-1}.
397 - Kinetic studies showed that the K_m of lipase increased from 1.63 mM to 4.48 mM
398 after immobilization.
399 - The immobilized lipase maintained 85% of its original activity after the same wool
400 immobilized lipase was used in six consecutive tributyrin hydrolysis reactions in the
401 BSTR. This result is superior to previous wool immobilized enzyme systems,
402 therefore taking immobilized lipase systems one step closer to implementation in
403 continuous enzyme reaction technologies. Further studies are needed to determine the
404 effect of truly continuous operation.
405 - The immobilized lipase displayed good storage stability, maintaining 75.8% of the

initial activity after storage of 40 weeks in buffer at 4 °C.

These results show that the wool immobilized lipase can produce a highly active and stable performance for tributyrin hydrolysis in a conventional BSTR. To extend these results to another reactor system, the immobilized lipase was successfully applied in tributyrin emulsion hydrolysis in an innovative enzyme process intensification technology: the SCDR. This reactor appears to intensify the reaction compared to the BSTR results (under comparable conditions), indicating that the combination of mesh and/or cloth immobilized enzymes and SCDRs is a very promising system for improving enzyme reactions in future applications.

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535 Figure 1 (a) Schematic diagram of the enzymatic reactor system with the SCDR. (b) Top
536 view of a woolen cloth with immobilized lipase on the disc of the SCDR.

537 Figure 2 Effect of lipase amount in the immobilization solution on the enzyme loading and
538 activity of the immobilized lipase. The immobilized lipase was cross-linked with 0.1% GA.
539 Values are the average of three independent replicates; error bars represent average ± one
540 standard deviation.

541 Figure 3 Effect of reaction pH on the activity of free and immobilized lipase. Values are the
542 average of three independent replicates; error bars represent average ± one standard
543 deviation.

544 Figure 4 Effect of reaction temperature on the activity of free and immobilized lipase. Values
545 are the average of three independent replicates; error bars represent average ± one standard
546 deviation.

547 Figure 5 Thermal stability of free and immobilized lipase at 60°C. Values are the average of
548 three independent replicates; error bars represent average ± one standard deviation.

549 Figure 6 Thermal deactivation of immobilized lipase at various temperatures. Values are the
550 average of three independent replicates; error bars represent average ± one standard
551 deviation.

552 Figure 7 Arrhenius plot of deactivation rate constant (k_d)

553 Figure 8 Lineweaver-Burk plots of free and immobilized lipase in tributyrin hydrolysis
554 showing that Michaelis-Menton kinetics fit the initial rate data. Values are the average of
555 three independent replicates; error bars represent average ± one standard deviation.

556 Figure 9 Storage stability of the immobilized lipase on woolen cloth: the effect of
557 temperature on the residual activity when stored in phosphate buffer (pH 7, 0.1 M). Values

558 are the average of three independent replicates; error bars represent average ± one standard
559 deviation.

560 Figure 10 Operational stability of the immobilized lipase on woolen cloth: results for
561 repeated use of wool immobilized lipase in six consecutive tributyrin hydrolysis reactions in
562 the BSTR. Values are the average of three independent replicates.

563 Figure 11 Time course reaction data from the pH stat comparing tributyrin emulsion
564 hydrolysis in the BSTR and SCDR at different concentrations. Operational conditions of
565 SCDR are as follows: reactant volume of 1 L, reaction temperature of 45 °C, flow rate of 5
566 mL s^{-1} and spinning speed of 350 rpm. The same enzyme to substrate ratio was maintained in
567 comparing the SCDR and BSTR. Values are the mean of three independent replicates.

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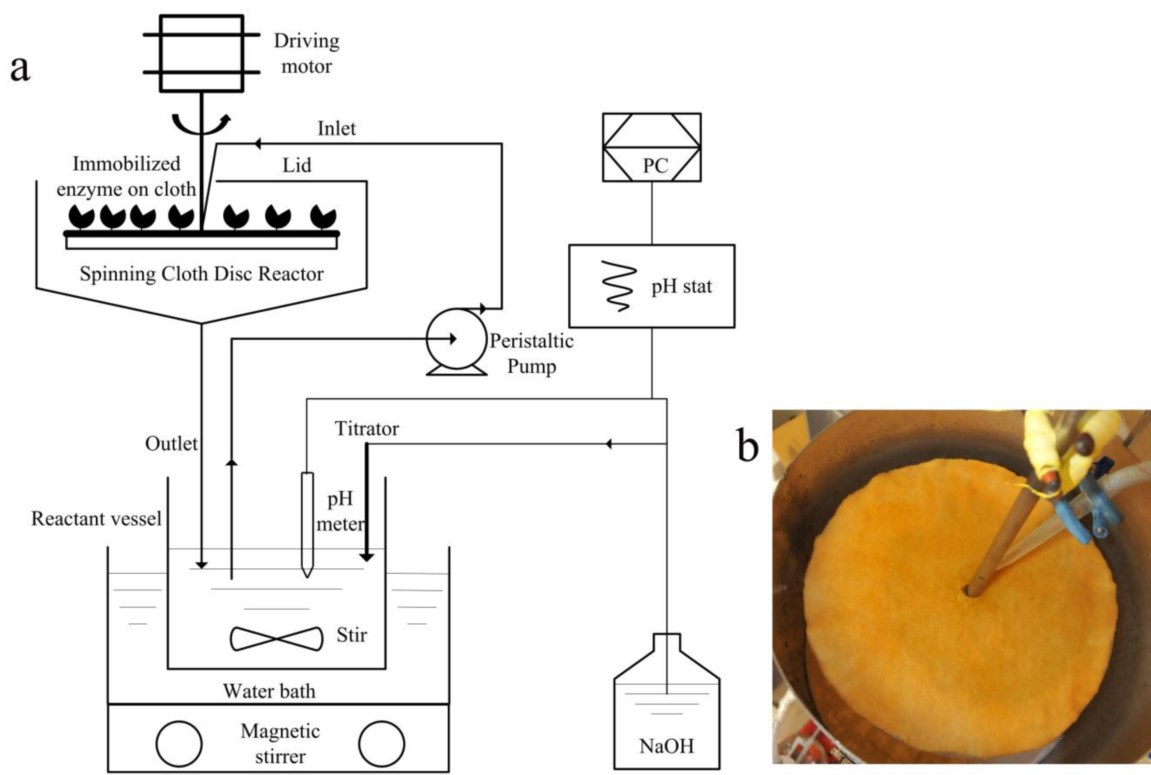

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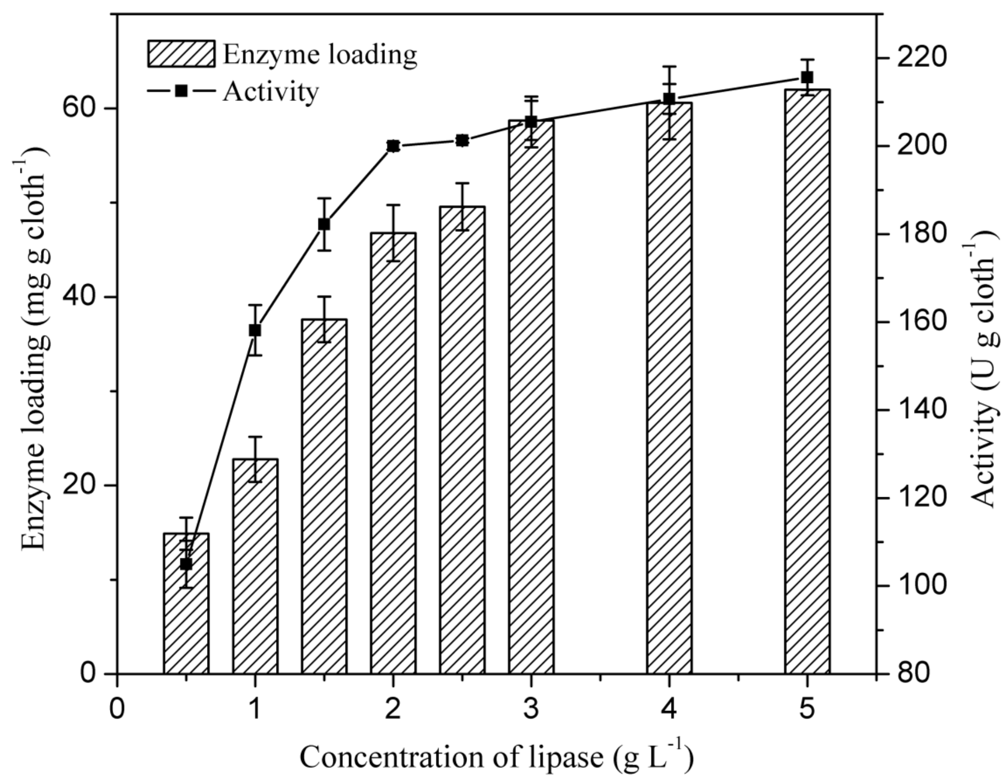

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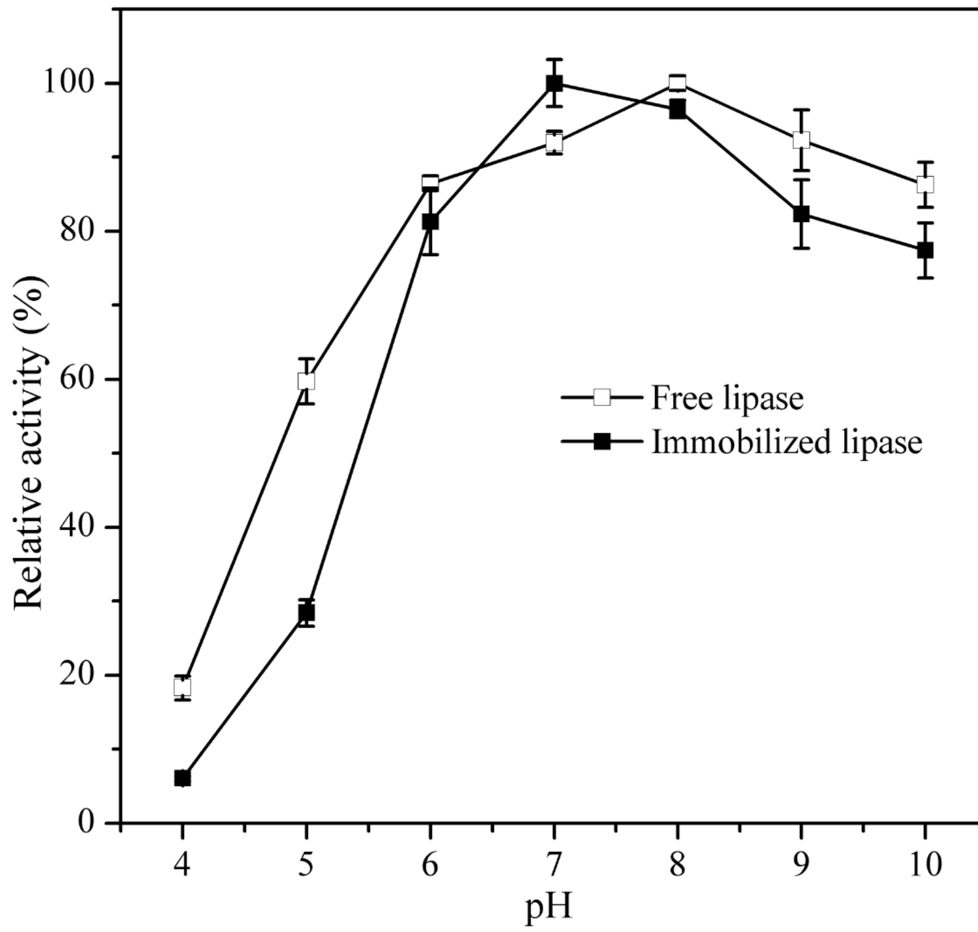

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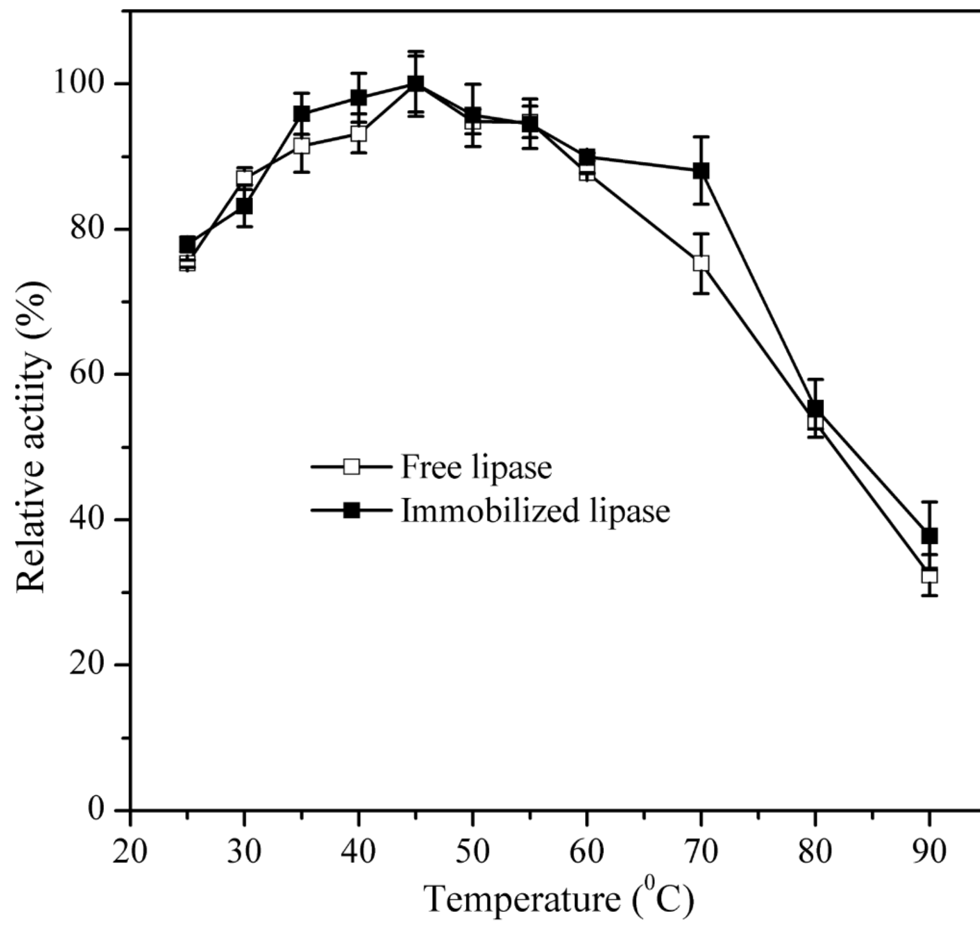

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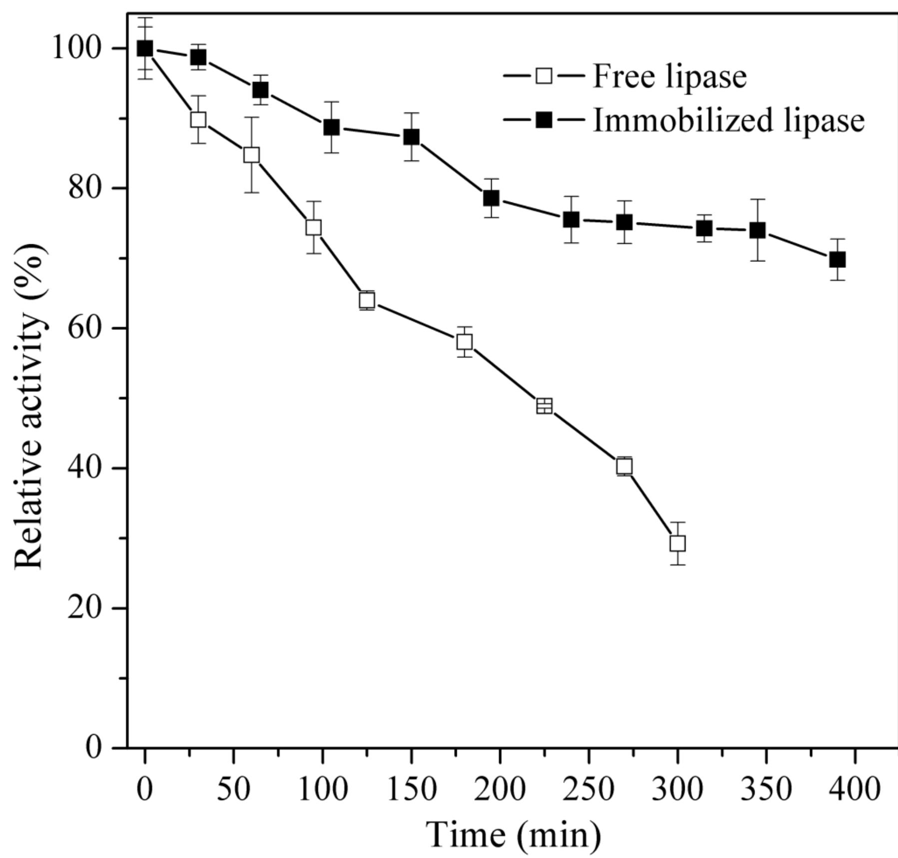

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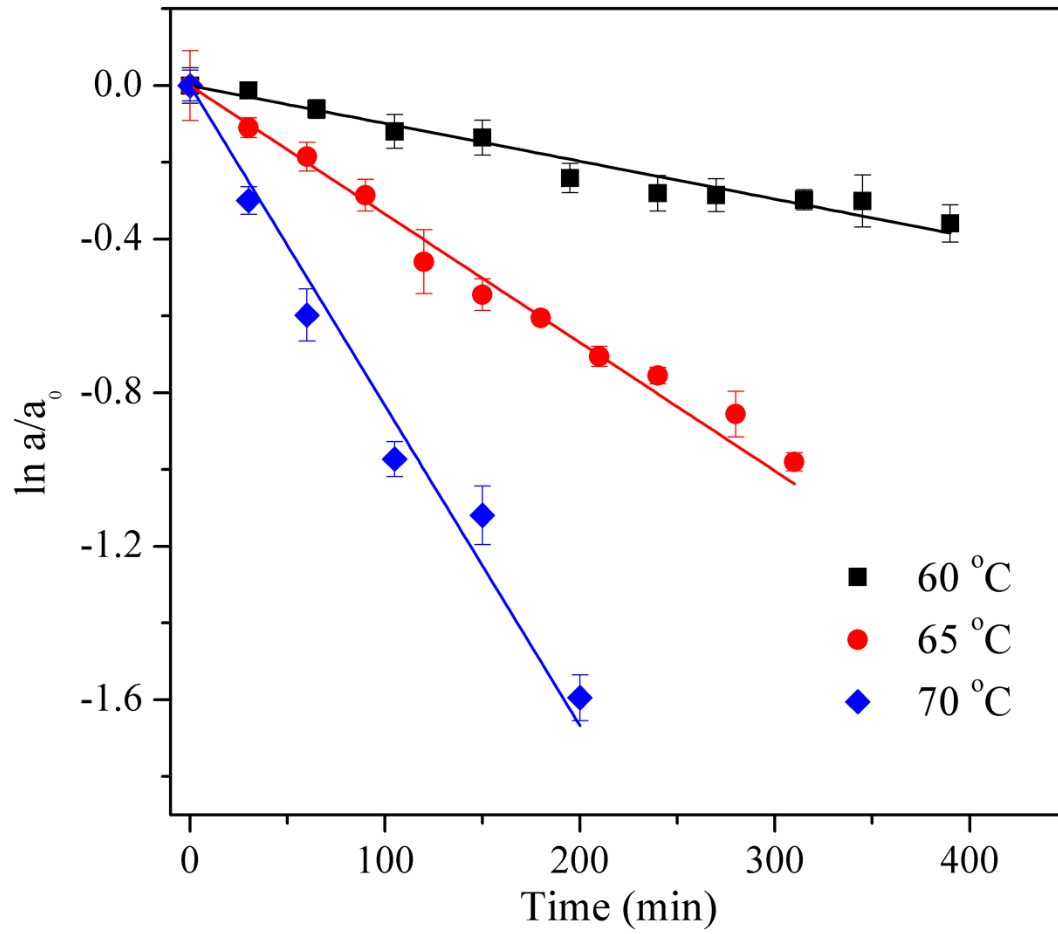

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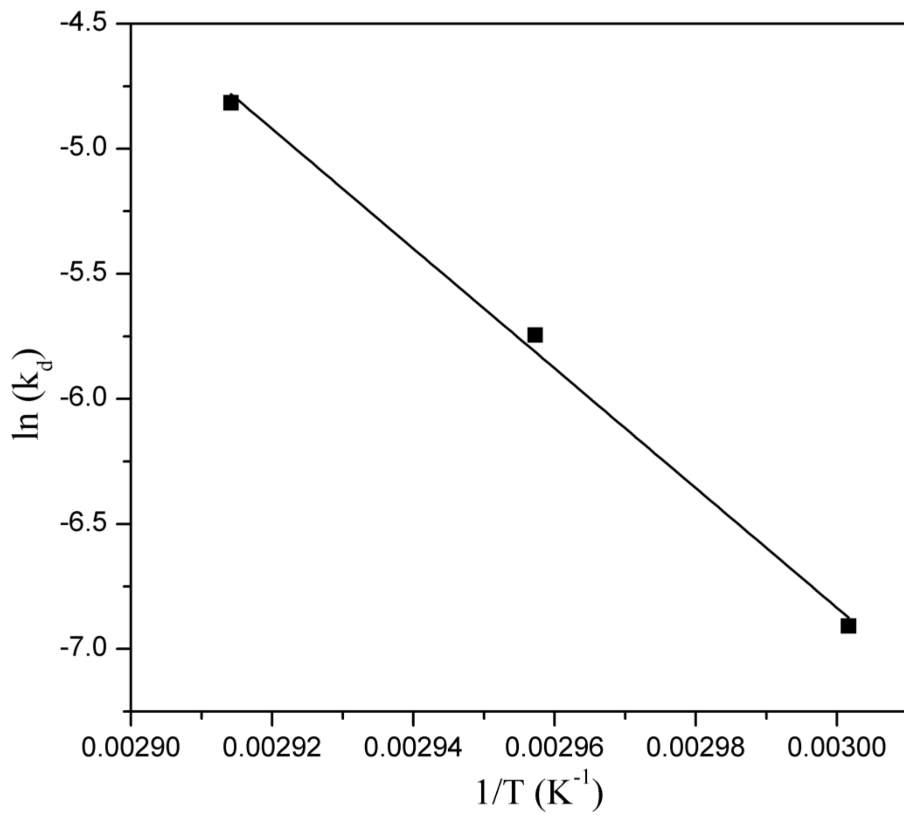

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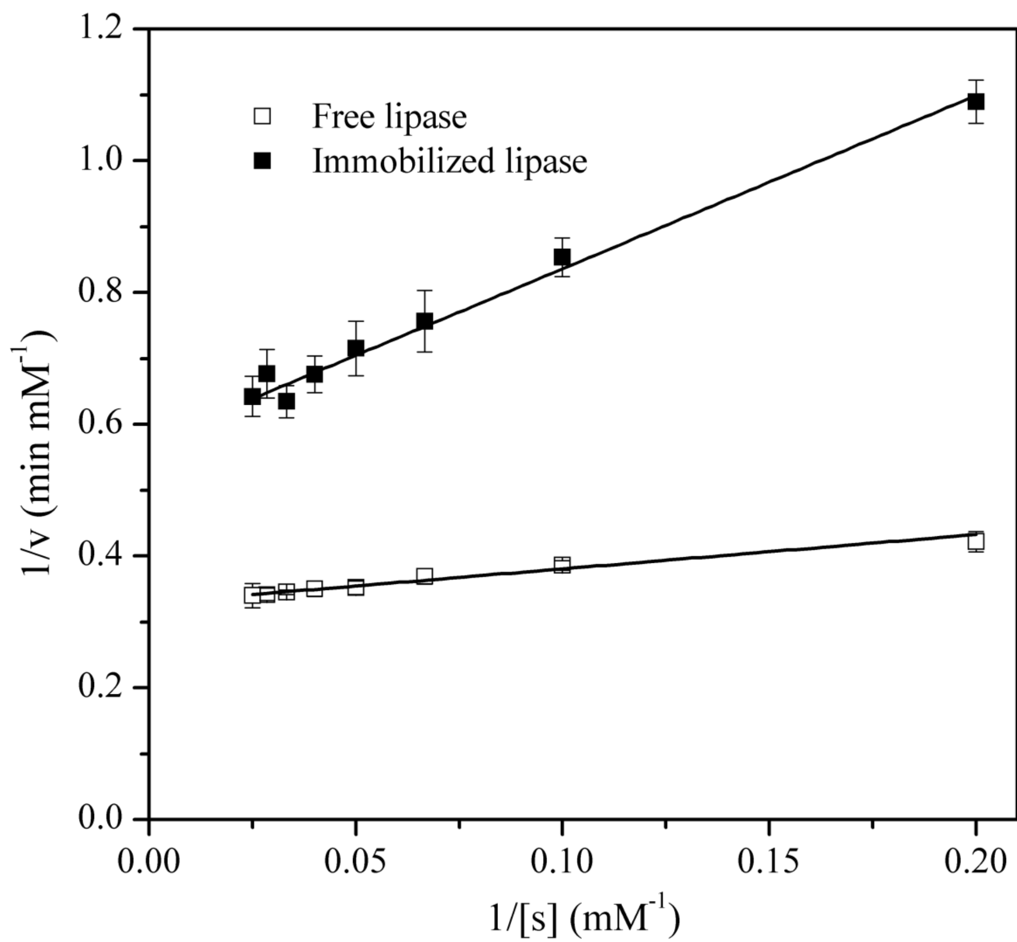

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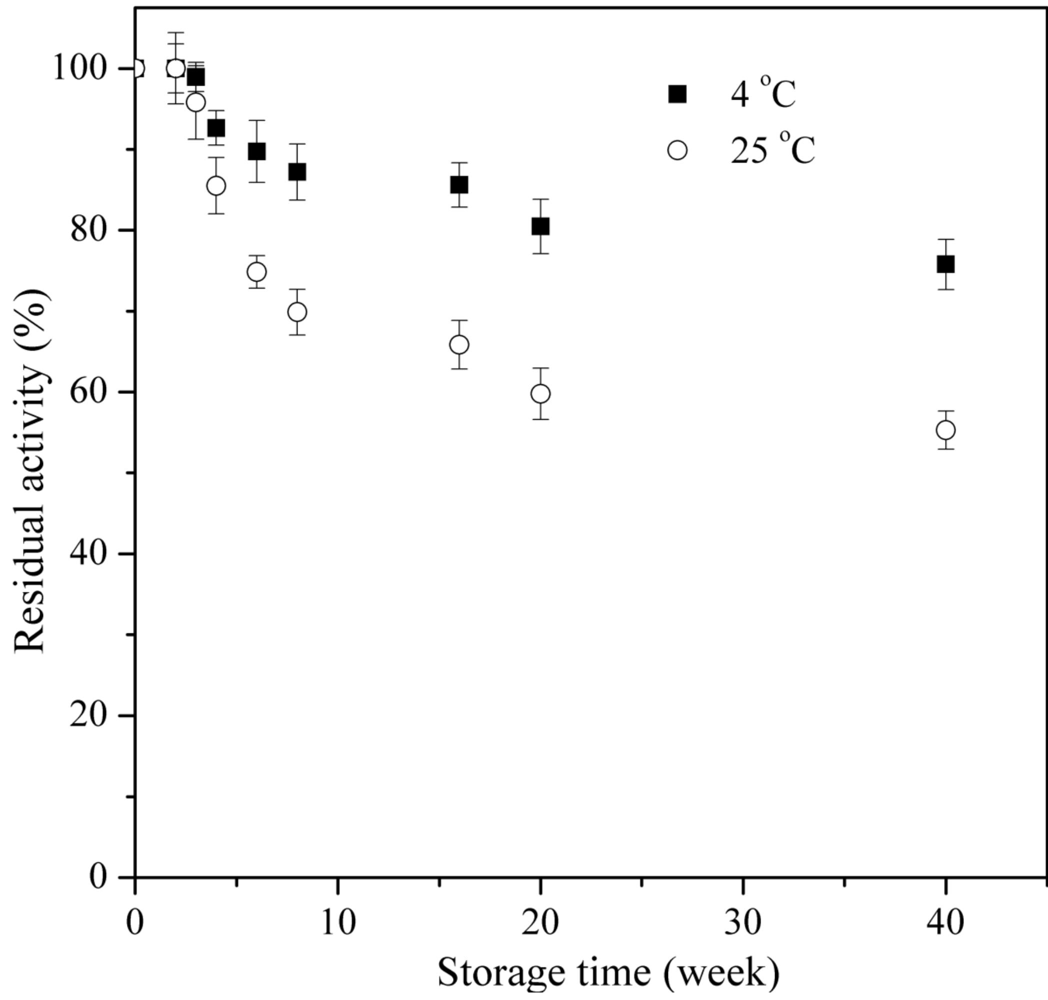

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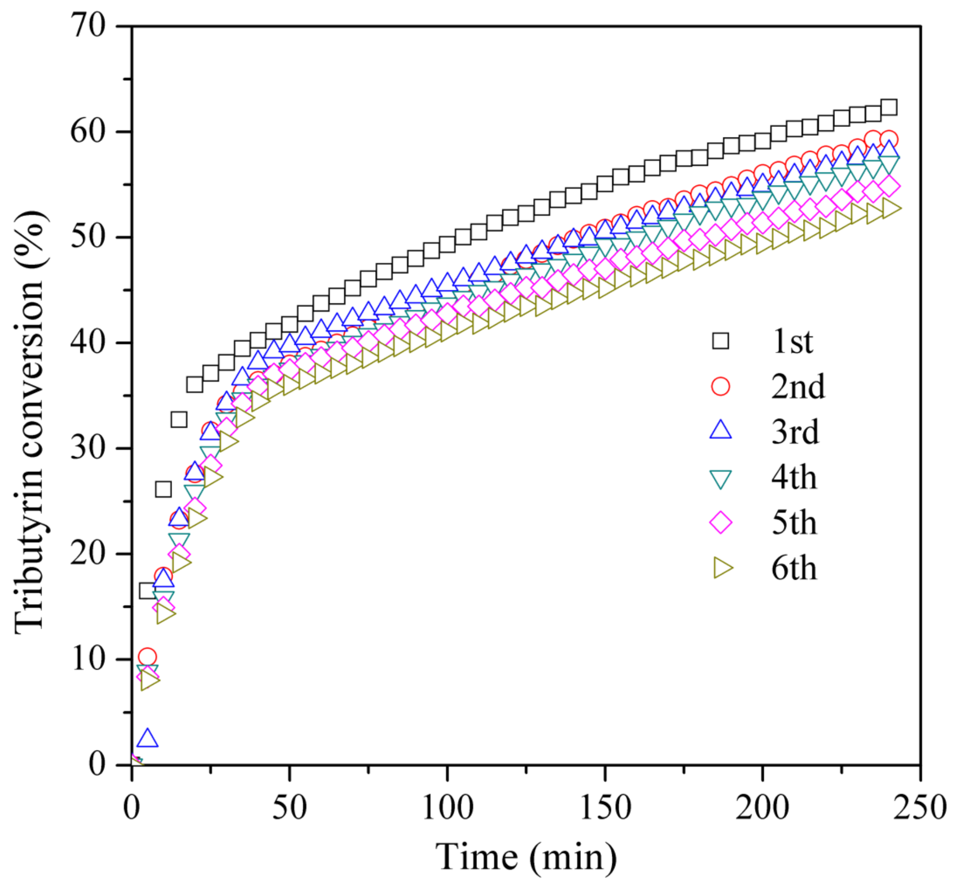

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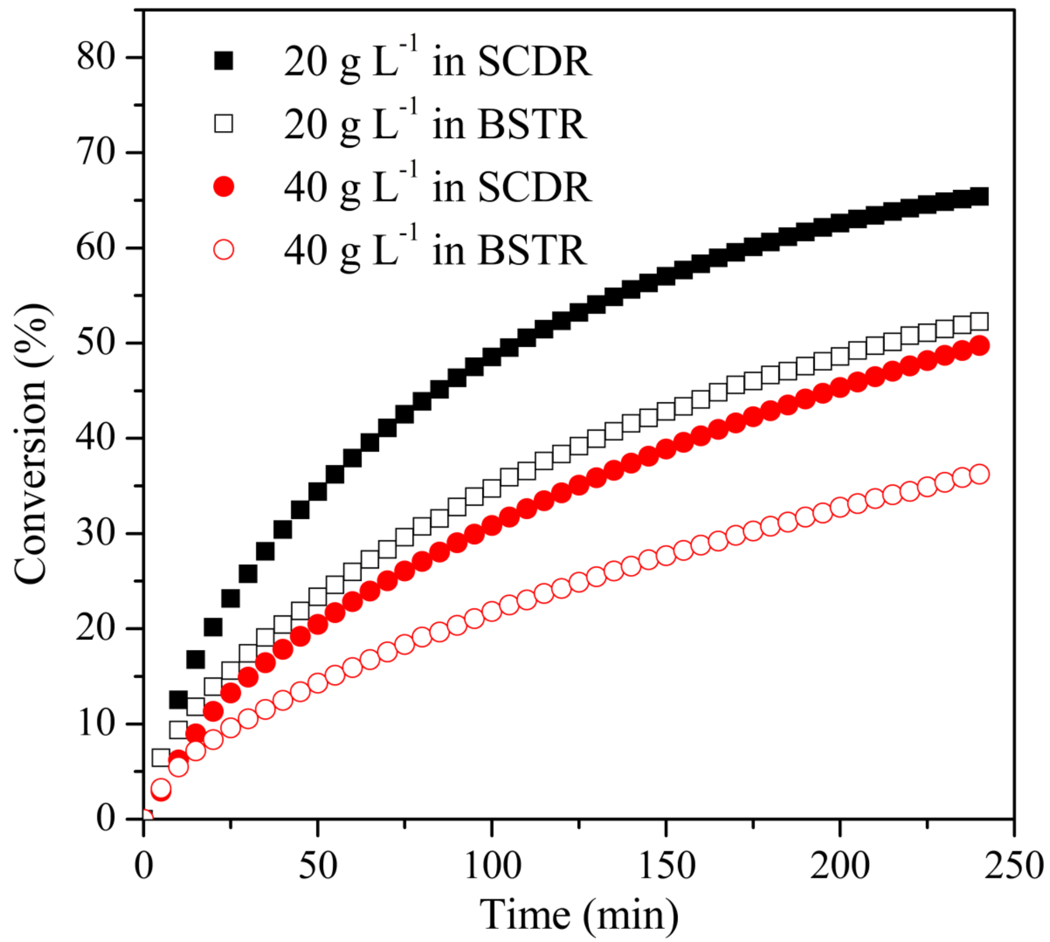

Figure 11

Table 1 The deactivation rate constant (k_d) and half-life ($t_{1/2}$) of immobilized lipase at various temperatures. Error bars are calculated as ± one standard deviation.

T (^oC)	k_d (min^{-1})	$t_{1/2}$ (min)
60	0.000985±0.00015	703.7±79.8
65	0.00335±0.0003	206.9±10.5
70	0.00834±0.00045	83.1±4.3